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The Proapoptotic Factors Bax and Bak Regulate T Cell Proliferation through Control of Endoplasmic Reticulum Ca^{2+} Homeostasis

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SUMMARY

The Bcl-2-associated X protein (Bax) and Bcl-2-antagonist/killer (Bak) are essential regulators of lymphocyte apoptosis, but whether they play a role in viable T cell function remains unclear. Here, we report that T cells lacking both Bax and Bak display defects in antigen-specific proliferation because of Ca^{2+}-signaling defects. *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells displayed defective T cell receptor (TCR)- and inositol-1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} mobilization because of altered endoplasmic reticulum (ER) Ca^{2+} regulation that was reversed by Bax's reintroduction. The ability of TCR-dependent Ca^{2+} signals to stimulate mitochondrial NADH production in excess of that utilized for ATP synthesis was dependent on Bax and Bak. Blunting of Ca^{2+}-induced mitochondrial NADH elevation in the absence of Bax and Bak resulted in decreased reactive-oxygen-species production, which was required for T cell proliferation. Together, the data establish that Bax and Bak play an essential role in the control of T cell proliferation by modulating ER Ca^{2+} release.

INTRODUCTION

Antigenic stimulation of T lymphocytes through the T cell receptor (TCR) initiates a coordinated program of activation, proliferation, and differentiation. This program of expansion and differentiation gives rise to a pool of activated T cells that function to control and clear foreign pathogens. Effective initiation of T lymphocyte activation is critical for proper function of the adaptive immune system (Ohashi, 2002). An equally important step in the T cell response is the elimination of excess effector T cells and the generation of a long-term memory T cell pool after pathogen clearance (Marsden and Strasser, 2003). Defects in apoptosis are the underlying cause of several lymphoproliferative syndromes in mice and humans (Bidere et al., 2006), suggesting that deregulated T cell survival after activation may lead to the accumulation of excess lymphocytes and the development of autoimmunity.

Members of the B cell leukemia/lymphoma-2 (Bcl-2) protein family play central roles in the regulation of T cell survival and apoptosis. Bcl-2-associated X protein (Bax) and Bcl-2-antagonist/killer (Bak)—proapoptotic members of this family—are required for the induction of mitochondrial-dependent apoptotic pathways in multiple tissue and cell types (Lindsten et al., 2000). Either Bax or Bak is required for cytochrome c release from mitochondria in response to apoptotic stimuli (Wei et al., 2000; Cheng et al., 2001), and cells lacking both Bax and Bak are resistant to cell death induced by a variety of stimuli including DNA damage, growth-factor withdrawal, and nutrient starvation (Wei et al., 2001). Antiapoptotic members of this family including Bcl-2 and Bcl-X_L antagonize the prodeath functions of Bax and Bak. Concurrent with this model, transgenic overexpression of Bcl-2 or Bcl-X_L in the T cell lineage is protective (Sentman et al., 1991; Strasser et al., 1991; Chao et al., 1995), whereas loss of these proteins through genetic deletion decreases the viability of developing and mature lymphocytes (Veis et al., 1993; Nakayama et al., 1994; Motoyama et al., 1995). *Bax*$^{-/-}$, *Bak*$^{-/-}$

animals display abnormalities in thymopoiesis and peripheral T cell homeostasis marked by enhanced survival of thymocytes and the development of splenomegaly and lymphadenopathy (Lindsten et al., 2000; Rathmell et al., 2002). Together, these data suggest that Bax and Bak are key regulators of T cell apoptosis and as such may modulate T cell accumulation during and after an immune response.

Ca^{2+} is an important second messenger involved in the control of T cell proliferation, apoptosis, differentiation, and metabolism. One of the key signaling events triggered by TCR engagement is the elevation of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). TCR-dependent intracellular Ca^{2+} flux occurs through two distinct steps. First, inositol-1,4,5-trisphosphate (IP_3) generated after TCR ligation promotes the release of Ca^{2+} from endoplasmic reticulum (ER) stores. Depletion of ER Ca^{2+} stores promotes the influx of Ca^{2+} from the extracellular environment through Ca^{2+}-release-activated Ca^{2+} (CRAC) channels (Venkatachalam et al., 2002). In addition to their role in mitochondrial physiology, Bcl-2 family members also function at the ER to modulate intracellular Ca^{2+} homeostasis. Although the data remain controversial, proteins of the Bcl-2 family have been shown to affect Ca^{2+} homeostasis through control of ER Ca^{2+} leak at least in part by modulating IP_3 receptor (IP_3R) activity (Chen et al., 2004; Oakes et al., 2005; White et al., 2005). Both proapoptotic and antiapoptotic members of the Bcl-2 family localize to the ER, and Bcl-X_L interacts directly with the IP_3R to modulate its sensitivity to IP_3 (Zong et al., 2003; White et al., 2005). Bcl-2 has been shown to bind to the IP_3R and negatively regulate Ca^{2+} flux (Chen et al., 2004). Transgenic overexpression of Bcl-2 or Bcl-X_L can affect Ca^{2+} mobilization in T cells (Linette et al., 1996; Li et al., 2002). Whether Bax or Bak plays a physiological role in the control of Ca^{2+} signaling in T cells has not been investigated.

The central role of Bax and Bak in the control of apoptosis is now well established. However, whether these molecules contribute to the normal physiology of viable peripheral T cells remains unclear. Here, we report that combined deficiency of Bax and Bak in lymphocytes leads to profound defects in T cell proliferation in vitro and in vivo. T cells deficient for both Bax and Bak displayed decreased $[Ca^{2+}]_i$ signaling in response to TCR stimulation, and this signaling could be restored through reintroduction of Bax. $Bax^{-/-}$, $Bak^{-/-}$ T cells displayed $[Ca^{2+}]_i$ oscillations of higher frequency but lower amplitude in response to TCR stimulation that resulted in an overall lower $[Ca^{2+}]_i$ signal relative to wild-type T cells. Restoration of Ca^{2+} signaling through addition of ionomycin rescued this proliferative defect. Bax and Bak were required for the Ca^{2+}-dependent production of mitochondrial NAD(P)H in response to TCR stimulation. This stimulation of mitochondrial NAD(P)H during T cell activation is in excess of the ability of the mitochondrial ATP synthase to convert the NADH into ATP and contributed to a stimulation of cellular reactive oxygen species (ROS) production. Ca^{2+}-dependent ROS production was inhibited in DKO T cells. Consistent with recent reports, normal T cell proliferation was blocked when TCR-dependent ROS signals were inhibited, even in the presence of elevated $[Ca^{2+}]_i$. Our studies demonstrate that Bax and Bak are critical regulators of T cell proliferation through regulation of TCR-dependent Ca^{2+} mobilization, mitochondrial bioenergetics, and ROS production.

RESULTS

Bax and Bak Are Essential for T Cell Proliferation and Apoptosis

Mice deficient for both Bax and Bak display developmental and homeostatic irregularities in multiple tissues including the hematopoietic system (Lindsten et al., 2000). To study the specific effect of combined deficiency of Bax and Bak on peripheral T cell function, we reconstituted lethally irradiated recombination-activating gene-1 (RAG-1)-deficient mice with bone marrow or fetal liver from $Bax^{-/-}$, $Bak^{-/-}$ or wild-type (WT) donors. T cells were isolated from the spleen and lymph nodes of $Bax^{-/-}$, $Bak^{-/-}$ or wild-type chimeric animals and stimulated with anti-CD3. $Bax^{-/-}$, $Bak^{-/-}$ T cells displayed defective proliferation in relation to control T cells (Figure 1A). The decreased proliferative response of $Bax^{-/-}$, $Bak^{-/-}$ T cells was most evident at lower concentrations (<5 μg/ml) of anti-CD3, suggesting that loss of Bax and Bak raises the threshold for T cell activation. The addition of anti-CD28 enhanced but did not correct the proliferative response of $Bax^{-/-}$, $Bak^{-/-}$ T cells (Figure 1B). As observed with conventional $Bax^{-/-}$, $Bak^{-/-}$ mice, $Bax^{-/-}$, $Bak^{-/-}$ chimeric mice developed homeostatic defects in the peripheral lymphoid organs (splenomegaly and lymphadenopathy) marked by an accumulation of resting T cells with a memory-like phenotype (Figure S1 in the Supplemental Data available online). However, the resting T cells that accumulate in $Bax^{-/-}$, $Bak^{-/-}$ chimeric mice displayed no substantial changes in their T cell repertoire (Figure S2). TCR-dependent proliferation of T cells from animals lacking either Bax or Bak alone was similar to wild-type cells, suggesting that Bax and Bak perform redundant functions with respect to T cell proliferation (Figure S3).

Analysis of T cell division by CFSE dye dilution revealed that $CD4^+$ T cells from $Bax^{-/-}$, $Bak^{-/-}$ chimeric animals displayed a profound delay in cell division in response to anti-CD3 treatment, with a majority of cells being unable to initiate cell division 3 days after activation (Figure 1C). This decrease in cell division was accompanied by a decreased ability to upregulate the T cell activation markers CD25 and CD69 in $Bax^{-/-}$, $Bak^{-/-}$ T cells in response to anti-CD3 treatment, despite a slightly increased basal expression of these markers in the resting $Bax^{-/-}$, $Bak^{-/-}$ T cells (Figure 1D). Normal upregulation of CD25 and CD69 could not be fully rescued by CD28 costimulation. Together, these data reveal a requirement for Bax and Bak in the control of T cell activation and proliferation in response to antigen-receptor stimulation.

To assess whether $Bax^{-/-}$, $Bak^{-/-}$ T cells remain resistant to apoptosis despite eliciting a defective proliferative response, we cultured wild-type and $Bax^{-/-}$, $Bak^{-/-}$ T cells in cytokine-free medium after activation, and cell

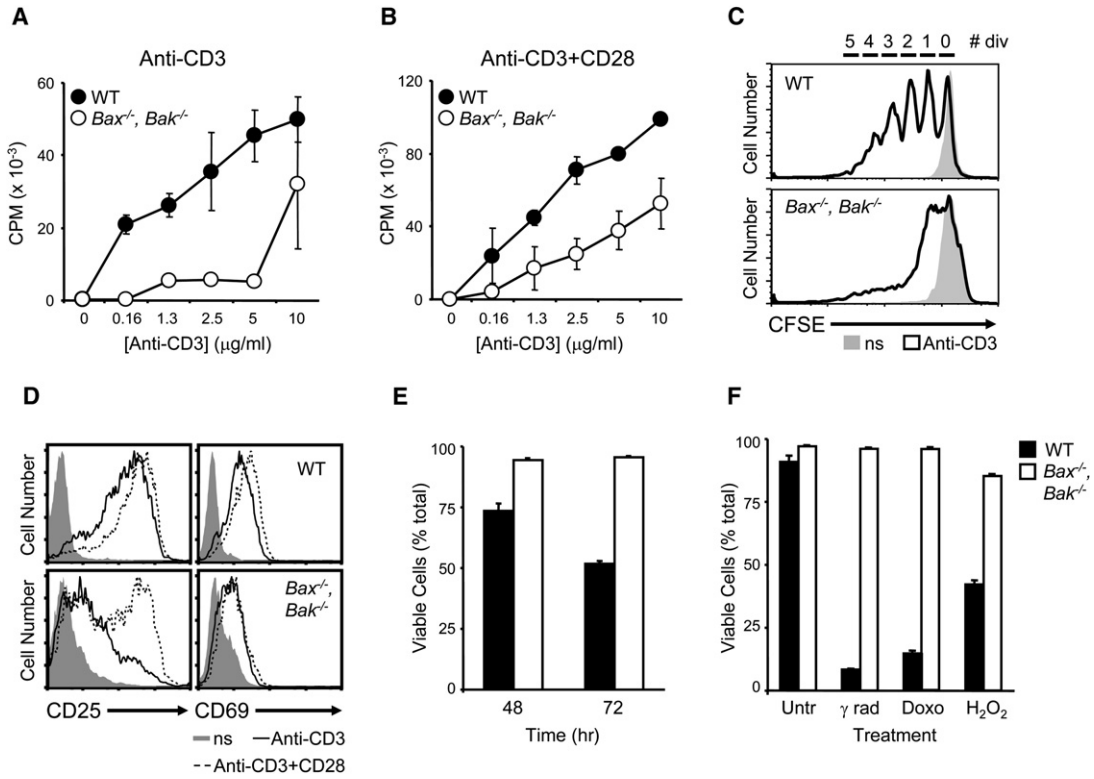

Figure 1. Bax and Bak Are Required for T Cell Activation and Apoptosis

(A and B) Proliferation of peripheral T cells isolated from *Rag1*$^{-/-}$ animals reconstituted with wild-type (WT, closed circles) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (open circles) bone marrow. T cells were stimulated with plate-bound anti-CD3 alone (A) or in combination with 1 μg/ml anti-CD28 (B), and proliferation was measured on day 3 postactivation. ^{3}[H]-thymidine was added during the last 12 hr of culture. Each data point represents the mean ± SD for triplicate cultures and is representative of four independent experiments.

(C) CFSE profile of wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ CD4^{+} T cells as measured by flow cytometry 3 days after activation with soluble anti-CD3 (0.5 μg/ml). Data are representative of three independent experiments.

(D) Surface expression of CD25 and CD69 on wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ CD4^{+} T cells one day after activation with anti-CD3 (solid line) or anti-CD3 plus anti-CD28 (broken line) as determined by flow cytometry. CD25 and CD69 surface staining of unstimulated cells is depicted by the gray histogram. Data are representative of two independent experiments.

(E) T cell viability under conditions of cytokine withdrawal. Wild-type (filled bars) and *Bax*$^{-/-}$, *Bak*$^{-/-}$ (open bars) T cells were activated with anti-CD3 and anti-CD28 for 3 days, then washed and replated in fresh medium with no exogenous cytokines. Cell viability was measured by exclusion of propidium iodide (PI) by flow cytometry. Data represent the mean ± SD for samples in triplicate from one of three independent experiments.

(F) T cell viability in response to apoptotic stimuli. Wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells were activated as shown in (E) and then subjected to the following various apoptotic stimuli: γ radiation (500 rad), doxorubicin (2 μg/ml), or H_2O_2 (100 μM). "Untr" stands for untreated. Percent viable cells was determined by PI exclusion and expressed as mean ± SD for triplicate cultures.

viability was measured over time. As seen in Figure 1E, wild-type T cells progressively lost viability in the absence of cytokine stimulation, whereas *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells remained viable. Activated *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells also maintained viability when challenged with various apoptotic stimuli including γ radiation, doxorubicin, and hydrogen peroxide (Figure 1F). Thus, deficiency of Bax and Bak confers T cell resistance to apoptosis despite a block in antigen-specific proliferation.

Bax and Bak Control T Cell Responsiveness In Vivo

To address whether deficiency of Bax and Bak affects T cell responsiveness in the context of a normal adaptive immune response, we infected wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric animals with a recombinant variant of the Gram-positive bacterium *L. monocytogenes* expressing OVA (rLmOVA) (Foulds et al., 2002). Seven days after infection with rLmOVA, spleens from infected wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ animals were analyzed for the expansion of H-2K^{b}-restricted OVA-specific CD8^{+} T cells. Expansion of OVA-specific T cells was observed in control chimeric mice but was severely impaired in *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeras (Figure 2A). This impairment was not due to slower kinetics for CD8^{+} T cell expansion in *Bax*$^{-/-}$, *Bak*$^{-/-}$ mice because *Bax*$^{-/-}$, *Bak*$^{-/-}$ mice failed to generate a significant number of OVA-specific T cells over the course of rLmOVA infection (Figure 2B). Moreover, loss of Bax and Bak crippled effector-cytokine production by CD8^{+} and CD4^{+} T cells in response to rLmOVA infection (Figure 2C and data not shown). Collectively, these data suggest

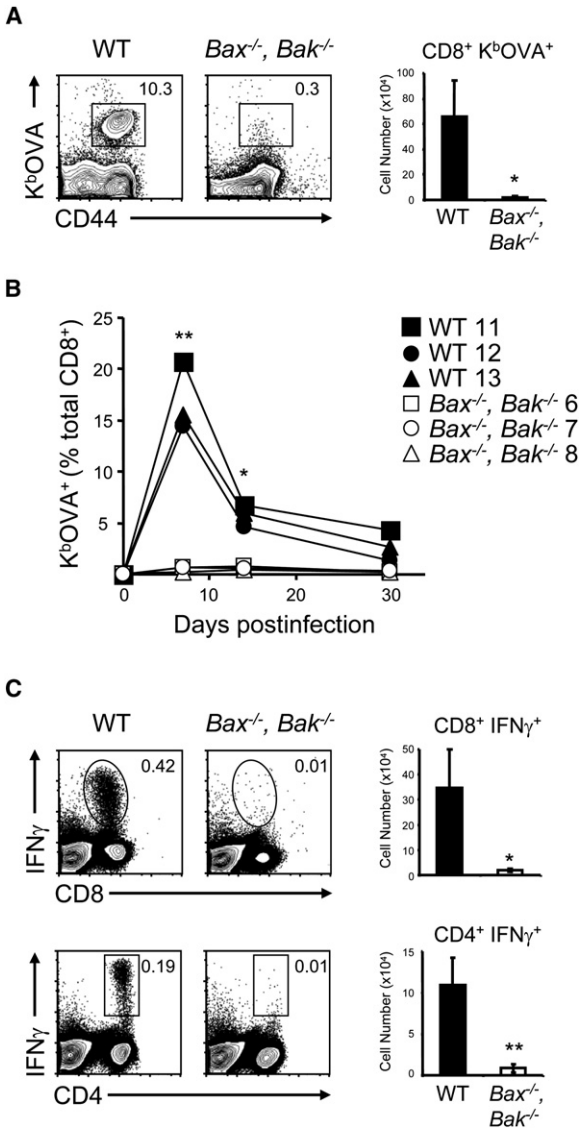

Figure 2. Impaired In Vivo T Cell Responses to *L. monocytogenes* Infection in *Bax*$^{-/-}$, *Bak*$^{-/-}$ Chimeric Mice

(A) Flow cytometry of splenocytes from wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric animals 7 days after i.v. infection with recombinant *L. monocytogenes* expressing OVA (rLmOVA, 5e^4 CFU). Numbers above gate indicate the percentage of OVA-specific cells as determined by tetramer staining on CD8$^+$ cells. The bar graph indicates the total number of $K^bOVA_{257-264}$-specific CD8$^+$ cells per wild-type (closed bar) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (open bar) spleen. The data represent the mean ± SEM of three animals per genotype and are representative of one of three independent experiments.

(B) Percentage of OVA-specific CD8$^+$ T cells in blood of wild-type (closed symbols) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (open symbols) animals over time after infection with rLmOVA as determined by tetramer staining and flow cytometry. Each symbol represents one mouse.

(C) Cytokine production as assessed by intracellular staining (ICS) and flow cytometry. Splenocytes from day 7 infected wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ animals were restimulated with $OVA_{257-264}$ or $LLO_{190-201}$ peptides (for CD8$^+$ and CD4$^+$ T cells, respectively) in the presence of GolgiStop and examined for IFNγ production by ICS and flow cytometry. Numbers above gates indicate percentage of IFNγ-producing cells in spleen. Bar graphs indicate the total number of IFNγ-producing CD8$^+$ and CD4$^+$ T cells per spleen. The data represent the mean ± SEM of three animals from one of three independent experiments. Statistical significance was determined by paired Student's t test (*$p < 0.05$, **$p < 0.01$).

that Bax and Bak are required for T cell proliferation and effective cytokine production by T cells against bacterial pathogens in vivo.

Defective TCR-Dependent Ca^{2+} Signaling in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells

TCR engagement initiates an organized cascade of signaling events that promote the transition of naive or memory T cells to activated effector T cells. For investigating possible mechanisms underlying the T cell proliferation defect observed in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, the activation of various T cell signaling pathways in wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells was examined after TCR activation. TCR-induced intracellular Ca^{2+} mobilization was dramatically decreased after anti-CD3 crosslinking of *Bax*$^{-/-}$, *Bak*$^{-/-}$ CD4$^+$ T cells relative to wild-type controls (Figure 3A), suggesting a role for Bax and Bak in the control of TCR-regulated Ca^{2+} homeostasis. No marked difference in other TCR-triggered signaling events, including extracellular-receptor-activated kinase (ERK) phosphorylation, NF-κB activation, or tyrosine phosphorylation, were observed after anti-CD3 stimulation (Figure 3B, and data not shown).

Increased $[Ca^{2+}]_i$ after TCR engagement is mediated by the generation of IP_3, which promotes Ca^{2+} release from the ER through activation of IP_3R Ca^{2+} channels (Lewis, 2001). Depletion of ER Ca^{2+} stores in turn promotes extracellular Ca^{2+} entry through CRAC channels in the plasma membrane, leading to a sustained increase in $[Ca^{2+}]_i$. TCR engagement promotes IP_3 generation by activating PLCγ1, an enzyme that converts membrane-associated PIP_2 to diacylglycerol (DAG) and IP_3. Phosphorylation of PLCγ1 was induced in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after TCR engagement (Figure 3C), suggesting the Ca^{2+}-signaling defect in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells occurred downstream of IP_3 generation. To assess the responsiveness of wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells to IP_3 directly, we measured $[Ca^{2+}]_i$ after treatment with a membrane-permeable IP_3 ester (Chen et al., 2004). Elevation of $[Ca^{2+}]_i$ by the IP_3 ester was decreased in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells in relation to controls, with fewer than 50% of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells responding after treatment (Figure 3D).

The observed decrease in TCR- and IP_3-inducible Ca^{2+} fluxes in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells may be due to impaired Ca^{2+} release from ER stores. For testing this possibility, wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells were stimulated by anti-CD3 crosslinking in the absence of extracellular Ca^{2+}, and the increase in $[Ca^{2+}]_i$ due to intracellular Ca^{2+} release was measured by flow cytometry. TCR stimulation induced an increase in $[Ca^{2+}]_i$ in wild-type cells, and such an increase was absent in *Bax*$^{-/-}$, *Bak*$^{-/-}$ cells, suggesting a defect in TCR-induced Ca^{2+} release from intracellular stores (Figure S4). Upon readdition of extracellular Ca^{2+}, wild-type cells displayed increased $[Ca^{2+}]_i$, suggesting

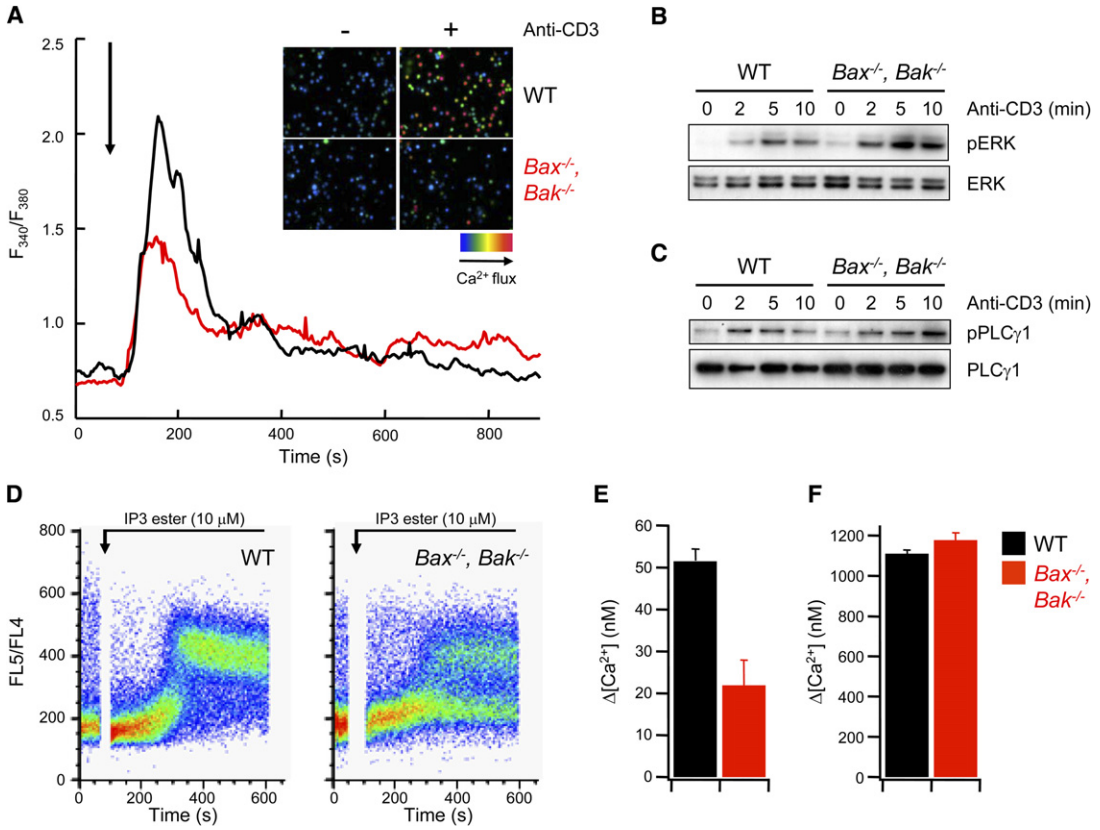

Figure 3. Impaired $[Ca^{2+}]_i$ Signaling in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells

(A) $[Ca^{2+}]_i$ of wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after TCR stimulation. $CD4^+$ T cells were incubated with fluo-4 dye and anti-CD3 antibody, washed, and plated on coverslips. Changes in $[Ca^{2+}]_i$ were measured by fluorescence microscopy after anti-CD3 crosslinking (indicated by arrow) and expressed as a function of time. The inset shows representative fields depicting $[Ca^{2+}]_i$ of wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells with (+) or without (–) anti-CD3 crosslinking.

(B and C) Immunoblot analysis of phosphorylated Erk (B) or PLCγ1 (C) in lysates from wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells stimulated for the indicated times with anti-CD3 (10 μg/ml).

(D) $[Ca^{2+}]_i$ of wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after stimulation with 10 μM IP_3 ester. T cells were loaded with indo-1 dye and $[Ca^{2+}]_i$ measured by flow cytometry.

(E and F) $[Ca^{2+}]_i$ of wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after thapsigargin (Tg) treatment. $[Ca^{2+}]_i$ of $CD4^+$ T cells was measured after application of Tg in Ca^{2+}-free buffer containing 1 mM EGTA (E) and measured again after switching to 1.8 mM Ca^{2+} solution (F). The data represent mean ± SEM for at least 90 cells from multiple trials.

influx of extracellular Ca^{2+} through open CRAC channels. This increase in $[Ca^{2+}]_i$ was blunted in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, suggesting that *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells exhibited incomplete activation of Ca^{2+} influx by TCR stimulation alone. Together, the data suggest that Bax and Bak act at the level of the ER to control release of intracellular Ca^{2+} stores in response to TCR-mediated IP_3 signals.

To assess ER Ca^{2+} content, we measured $[Ca^{2+}]_i$ of wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after treatment with thapsigargin (Tg). Tg acts to deplete ER Ca^{2+} stores by inhibiting the sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, which resequesters cytoplasmic Ca^{2+} to the ER in an ATP-dependent fashion (Thastrup et al., 1990). In the absence of extracellular Ca^{2+}, the Tg-stimulated increase in $[Ca^{2+}]_i$ was reduced in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells in relation to wild-type controls (Figure 3E and Figure S5), suggesting a reduced ER Ca^{2+} content in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. The decreased intracellular Ca^{2+} mobilization observed in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells in response to Tg was not due to increased Ca^{2+} efflux across the plasma membrane because Tg-dependent ER Ca^{2+} release was also reduced in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells treated with lanthanum chloride to inhibit plasma membrane Ca^{2+} pumps (Figure S5). Ca^{2+} influx after Tg-induced ER store depletion was similar between wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells (Figure 3F). Thus, Ca^{2+} influx in response to ER store depletion was normal in the absence of Bax and Bak. Collectively, these data demonstrate that deficiency of Bax and Bak in T cells lowers ER Ca^{2+} content. Reduced ER Ca^{2+} content and decreased IP_3-dependent Ca^{2+} release from the ER result in a reduced $[Ca^{2+}]_i$ response to TCR stimulation. Thus, the predominant defect in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells appears to be decreased IP_3-dependent Ca^{2+} release that is from intracellular stores and

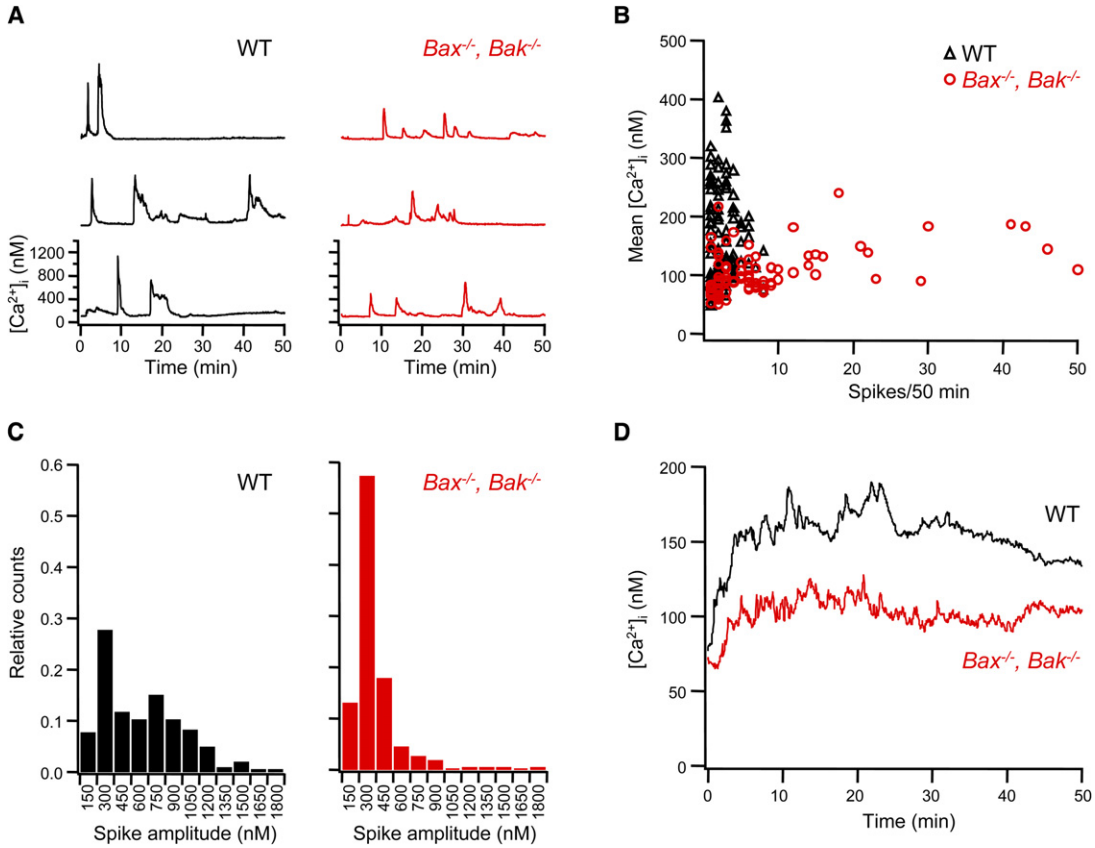

Figure 4. *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells Display Changes in TCR-Induced $[Ca^{2+}]_i$ Oscillations

(A) Representative traces of wild-type (black) and *Bax*$^{-/-}$, *Bak*$^{-/-}$ (red) T cells displaying $[Ca^{2+}]_i$ oscillations after stimulation with anti-CD3. $CD4^+$ T cells were loaded with fura-2 dye, plated onto anti-CD3 coated coverslips, and $[Ca^{2+}]_i$ measured by epifluorescence microscopy. Time t = 0 represents the point of contact between T cells and coverslips.

(B) A scatter plot showing the relationship between mean $[Ca^{2+}]_i$ for the spikes observed during a 50 min observation period and spike frequency for wild-type (black) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (red) T cells after activation with anti-CD3. Each data point represents the response of one cell.

(C) $[Ca^{2+}]_i$ spike amplitude histograms for wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after anti-CD3 stimulation.

(D) Average integrated $[Ca^{2+}]_i$ responses for wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after anti-CD3 stimulation. Data were calculated from traces of 96 wild-type and 87 *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells that responded to anti-CD3 with one or more $[Ca^{2+}]_i$ spikes over 50 min. The data are representative of four independent experiments.

that results in insufficient store depletion to fully activate CRAC channels in response to TCR ligation.

Bax and Bak Modulate TCR-Dependent Ca^{2+} Oscillations

The information conveyed by intracellular Ca^{2+} signals depends on the frequency and amplitude of $[Ca^{2+}]_i$ elevations. T cell receptor stimulation induces both transient and oscillatory $[Ca^{2+}]_i$ spikes (Donnadieu et al., 1992a; Donnadieu et al., 1992b). To assess the effect of combined deficiency of Bax and Bak on $[Ca^{2+}]_i$ dynamics within a single cell, we examined changes in $[Ca^{2+}]_i$ over time in individual T cells after TCR ligation. $CD4^+$ cells from wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric animals were loaded with fura-2 dye and activated with anti-CD3-coated coverslips. $[Ca^{2+}]_i$ signals were monitored over 50 min after anti-CD3 stimulation. Figure 4A depicts representative single-cell traces of $[Ca^{2+}]_i$ oscillations in wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after TCR ligation. Anti-CD3 stimulation induced transient $[Ca^{2+}]_i$ spikes in both wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells; however, *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells displayed a higher frequency of $[Ca^{2+}]_i$ spikes than wild-type cells. Approximately 20% of responding *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells displayed ten $[Ca^{2+}]_i$ spikes or more, whereas no wild-type cells responded with more than eight spikes over the same time period (Figure 4B). In contrast, the amplitudes of the $[Ca^{2+}]_i$ spikes induced by anti-CD3 stimulation were lower in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells relative to wild-type controls (Figure 4C). Consequently, the integrated $[Ca^{2+}]_i$ signal in response to TCR stimulation for the population of cells analyzed was diminished in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells compared with wild-type cells, with the maximal integrated $[Ca^{2+}]_i$ response of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells reaching only 30%–35% of that observed for wild-type cells (Figure 4D). Together, these data suggest that loss of Bax and Bak modifies the nature of

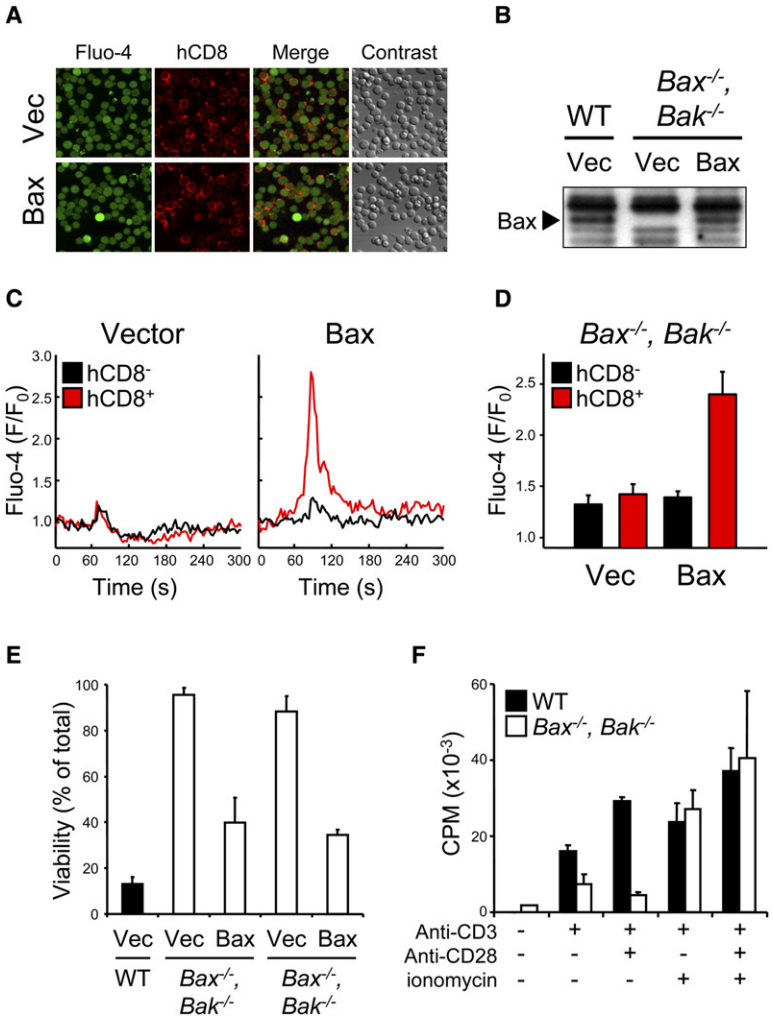

Figure 5. Re-expression of Bax Rescues the Ca^{2+} Mobilization Defect of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells

(A) Transduction of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells with Bax retrovirus. $CD4^+$ T cells from *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric mice were transduced with empty hCD8-Mig retrovirus (Vec) or retrovirus encoding Bax. Cells were incubated with fluo-4 dye, PE-tagged antibody against hCD8, and anti-CD3, washed, and plated on coverslips. A representative field of view from confocal imaging is displayed.

(B) Western blot of Bax expression in wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ $CD4^+$ T cells transduced with empty hCD8-Mig retrovirus (Vec) or retrovirus encoding Bax.

(C) $[Ca^{2+}]_i$ of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells re-expressing Bax. Changes in $[Ca^{2+}]_i$ were measured by confocal microscopy after anti-CD3 crosslinking and expressed as a function of time. The traces represent the $[Ca^{2+}]_i$ for the population of nontransduced ($hCD8^-$, black) and transduced ($hCD8^+$, red) cells within one field of view.

(D) Quantitation of peak $[Ca^{2+}]_i$ after anti-CD3 crosslinking for T cells in (C). The data represent the mean ± SEM for T cells from three independent mice.

(E) Viability of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells re-expressing Bax after cytokine withdrawal. Wild-type (filled bars) and *Bax*$^{-/-}$, *Bak*$^{-/-}$ (open bars) T cells transduced with empty hCD8-Mig retrovirus (Vec) or retrovirus encoding Bax were cultured in medium containing no exogenous cytokines. Cell viability was determined by trypan-blue exclusion 72 hr after cytokine withdrawal for T cells from two independent *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric mice. The data represent the mean ± SD for samples in triplicate.

(F) Ionomycin rescues the proliferative defect of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. Proliferation of wild-type (closed bars) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (open bars) T cells stimulated with anti-CD3 or anti-CD3 and anti-CD28 (1 μg/ml) in the presence or absence of ionomycin (250 ng/ml) is shown. Proliferation was measured 3 days after stimulation by ^{3}[H]-thymidine incorporation and expressed as mean ± SD. The data are representative of three independent experiments.

TCR-stimulated $[Ca^{2+}]_i$ signals: TCR stimulation triggers higher-frequency but lower-amplitude $[Ca^{2+}]_i$ oscillations in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, leading to an overall reduction in the integrated antigen-stimulated $[Ca^{2+}]_i$ signal.

Re-expression of Bax Restores Ca^{2+} Signaling in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells

One possible explanation for the unresponsiveness of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells is that lymphocyte development has been altered as a result of the combined deficiency of Bax and Bak, leading to the selection of mature T cells with an altered threshold for Ca^{2+} signaling. Although resistance to apoptosis in *Bax*$^{-/-}$, *Bak*$^{-/-}$ thymocytes leads to alterations in thymic cellularity in vivo (Rathmell et al., 2002), no substantial difference in the V_β repertoire of peripheral T cells from wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric animals was observed (Figure S2). To address whether the defect in $[Ca^{2+}]_i$ signaling was due to a cell-intrinsic requirement for Bax or Bak, we assessed TCR-induced $[Ca^{2+}]_i$ signaling in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after reintroduction of Bax. *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells were transduced with control retrovirus (hCD8-Mig) or retrovirus encoding cDNA for mouse Bax. The hCD8-Mig retrovirus drives the coexpression of a cDNA of interest and a functionally inert human CD8 biomarker, which permitted the identification of transduced T cells within the population (Figure 5A). *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells transduced with Bax retrovirus expressed Bax protein to a similar degree as that observed in wild-type T cells (Figure 5B). The effect of Bax re-expression on $[Ca^{2+}]_i$ signaling in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells was measured after TCR stimulation by confocal microscopy. TCR-induced $[Ca^{2+}]_i$ mobilization was restored in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells re-expressing Bax, whereas *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells transduced with the control vector remained unresponsive (Figures 5C and 5D). Importantly, hCD8-negative cells from Bax-transduced cultures continued to display defective $[Ca^{2+}]_i$ mobilization, indicating that the defect in TCR-dependent $[Ca^{2+}]_i$ signaling in

Bax$^{-/-}$, *Bak*$^{-/-}$ T cells is due to the cell-intrinsic absence of Bax.

To assess whether introduction of Bax could affect the sensitivity of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells to apoptosis, we transduced *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells with control or Bax-expressing retrovirus and measured the viability of transduced cells (hCD8^{+}) cultured in the absence of exogenous cytokines. As seen in Figure 5E, *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells expressing Bax displayed decreased viability in response to cytokine withdrawal, whereas *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells transduced with control vector remained viable. *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells expressing Bax also displayed decreased viability when treated with γ-radiation or doxorubicin (data not shown).

Ionomycin Corrects the Proliferative Defect Exhibited by *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells

We next examined whether the impaired proliferative response of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells could be solely attributed to the underlying defect in Ca^{2+} signaling. To address this issue, we examined the ability of the Ca^{2+} ionophore ionomycin to restore the proliferative response of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. Wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells were stimulated with anti-CD3 alone or in concert with anti-CD28 in the presence or absence of ionomycin, and proliferation was measured after 48 hr of culture by ^{3}[H]-thymidine incorporation. TCR ligation in the presence of ionomycin resulted in equivalent levels of proliferation of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells in comparison to wild-type T cells (Figure 5F). Costimulation through CD28 augmented the proliferative response of both populations to an equivalent extent when ionomycin was present.

Bax- and Bak-Dependent Ca^{2+} Flux Stimulates Mitochondrial Bioenergetics

Ca^{2+} release from the ER can enhance mitochondrial bioenergetics by stimulating enzymes of the tricarboxylic acid (TCA) cycle to generate ATP and other metabolic intermediates (Krauss et al., 2001). One consequence of Ca^{2+}-dependent stimulation of TCA cycle dehydrogenases is an elevation of mitochondrial NADH. NADH acts as an electron donor during mitochondrial respiration; electrons are transferred from NADH to complex I of the mitochondrial electron transport chain, a key step in ATP generation by mitochondria. To assess whether mitochondrial bioenergetics could be stimulated by T cell activation, we measured mitochondrial NAD(P)H fluorescence in CD4^{+} T cells after anti-CD3 crosslinking. TCR triggering promoted a rapid increase in NAD(P)H fluorescence in wild-type T cells (Figures 6A and 6B). Most of the NAD(P)H in response to TCR stimulation was generated through TCA-cycle production of NADH because the signal almost completely returned to baseline after addition of the mitochondrial electron transport uncoupling agent FCCP (Figure 6A). TCR-induced NADH accumulation was reduced in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, and the maximal NADH elevation approached only 30%–40% of controls (Figure 6B). To test whether Ca^{2+} signals could affect this response, we measured NAD(P)H fluorescence of WT

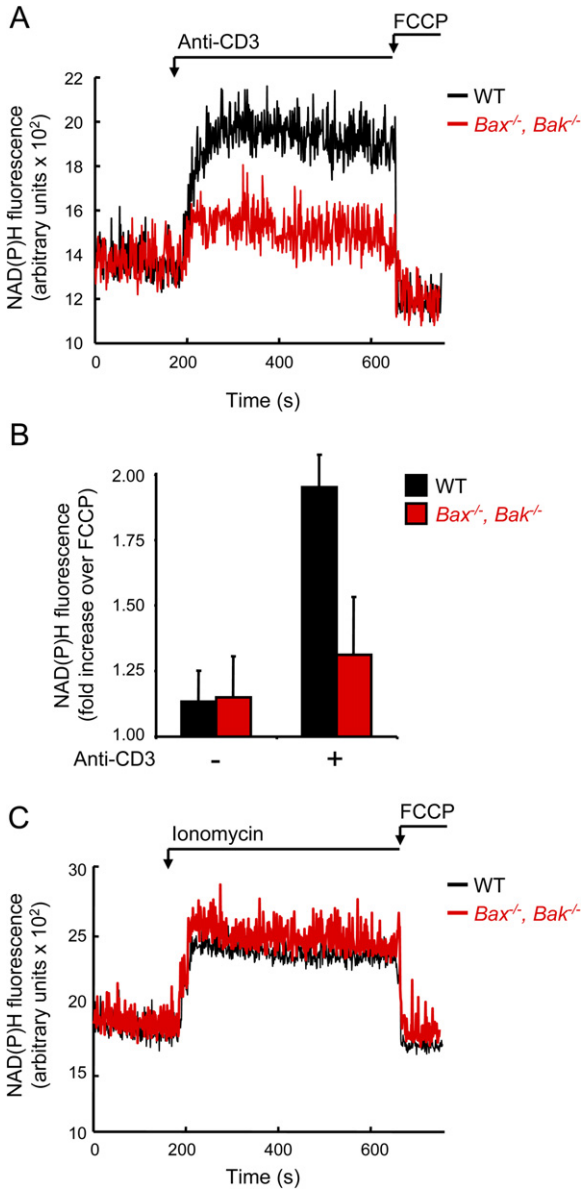

Figure 6. *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells Are Defective for TCR-Dependent Mitochondrial NADH Production

(A) NAD(P)H fluorescence of wild-type (black) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (red) T cells over time after anti-CD3 crosslinking. The mitochondrial uncoupling agent FCCP (1 μg/ml) was added as indicated.

(B) Mitochondrial NAD(P)H fluorescence before (–) or after (+) anti-CD3 crosslinking. Data are expressed as fold increase over FCCP-treated T cells and expressed as mean ± SD for triplicate samples.

(C) NAD(P)H fluorescence of wild-type (black) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (red) T cells over time after treatment with ionomycin (250 ng/ml). FCCP was added as indicated.

and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after ionomycin treatment. As seen in Figure 6C, ionomycin stimulated similar increases in NAD(P)H fluorescence in both wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. This Ca^{2+}-dependent NAD(P)H production was also due to increased mitochondrial NADH because FCCP completely collapsed the response.

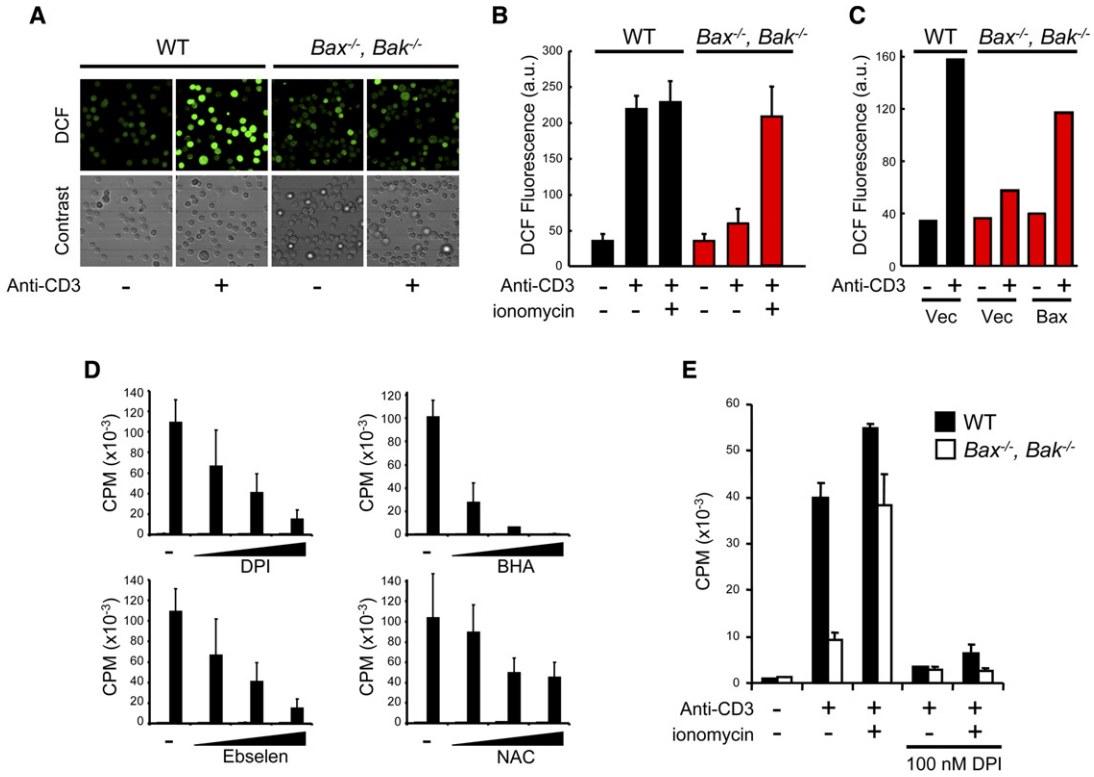

Figure 7. TCR-Induced ROS Generation Is Impaired in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T Cells

(A) Oxidation of the ROS-sensitive dye DCF-DA in wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells after anti-CD3 crosslinking. Top panels display representative fields of view for cells undergoing DCF oxidation (green) before (–) and after (+) anti-CD3 crosslinking. The phase-contrast view for the same field is shown.

(B) Quantification of DCF oxidation for wild-type (black) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (red) T cells after anti-CD3 treatment as in (A). ROS production by T cells was measured after anti-CD3 crosslinking alone or in combination with ionomycin (250 ng/ml). The data represent the mean ROS intensity ± SD in arbitrary units (a.u.) for triplicate samples and are representative of three independent experiments.

(C) Bax re-expression restores TCR-induced ROS production by *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. Quantification of DCF dye oxidation after anti-CD3 crosslinking in wild-type (black) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (red) T cells transduced with empty vector (Vec) or Bax. The data are representative of two independent experiments.

(D) Proliferation of T cells in the presence of inhibitors of ROS production. Purified T cells from wild-type animals were left unstimulated (open bars) or stimulated with anti-CD3 and anti-CD28 (closed bars) in the presence of increasing concentrations of DPI, BHA, Ebselen, or NAC. Proliferation was measured by ^{3}[H]-thymidine incorporation 48 hr after activation. The data represent the mean ± SD for triplicate samples and are representative of three independent experiments.

(E) Proliferation of wild-type (black) or *Bax*$^{-/-}$, *Bak*$^{-/-}$ (white) T cells stimulated with anti-CD3 or anti-CD3 plus ionomycin in the presence or absence of DPI (100 nM). Data are expressed as mean ± SD for triplicate samples and are representative of two independent experiments.

Bax and Bak Are Required for TCR-Dependent ROS Production

One predicted consequence of increased mitochondrial NADH levels is the generation of cellular ROS from a saturated electron transport chain. ROS are produced in T cells in response to TCR stimulation, a process that depends on TCR-mediated Ca^{2+} signaling (Devadas et al., 2002). To determine whether TCR-induced ROS production is influenced by Bax and Bak, we stimulated wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells by anti-CD3 crosslinking in the presence of the redox-sensitive dye DCF-DA and measured dye oxidation by confocal microscopy. TCR stimulation rapidly induced ROS production in wild-type T cells but not *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells (Figure 7A). Approximately 80% of wild-type T cells responded to TCR stimulation with maximal DCF fluorescence, compared to 5% of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. However, *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells displayed a normal ROS response after addition of ionomycin (Figure 7B), suggesting that Ca^{2+} signals could rescue the defect in ROS production observed in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. Consistent with this possibility, re-expression of Bax restored TCR-induced ROS production in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, indicating a cell-intrinsic requirement for Bax in this process (Figure 7C).

To assess the impact of ROS production on T cell proliferation, we measured the proliferative responses of wild-type T cells cultured in the presence of several compounds known to inhibit the generation of TCR-dependent ROS: diphenylene iodonium (DPI), butylated hydroxyanisole (BHA), Ebselen, and N-acetyl-cysteine (NAC). Each compound induced a dose-dependent decrease in T cell proliferation (Figure 7D). To assess whether enhanced

Ca^{2+} signaling could bypass the requirement for ROS in T cell proliferation, we activated wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells by anti-CD3 and ionomycin stimulation in the presence of DPI. DPI is an inhibitor of flavoprotein dehydrogenases reported to block TCR-induced production of H_2O_2 and superoxide (Jackson et al., 2004). DPI inhibited the proliferation of wild-type and *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, even in the presence of ionomycin (Figure 7E), suggesting that the ROS-dependent signal that drives proliferation lies downstream of TCR-mediated effects on $[Ca^{2+}]_i$. Together, these data demonstrate that Bax and Bak contribute to the ability of TCR- and Ca^{2+}-dependent signaling to induce cellular ROS and subsequent T cell proliferation.

DISCUSSION

The proapoptotic Bcl-2 family members Bax and Bak are required regulators of mitochondrial-dependent apoptotic pathways in lymphocytes. Here, we demonstrate a nonapoptotic role for Bax and Bak in the control of T cell proliferation. *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells display defective proliferation in response to TCR and costimulatory signals in vitro and fail to proliferate or produce effector cytokines in response to the bacterial pathogen *L. monocytogenes* in vivo. TCR-dependent Ca^{2+} signaling is impaired in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells because of reduced ER Ca^{2+} release after TCR ligation. In comparison to wild-type cells, *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells displayed $[Ca^{2+}]_i$ oscillations that had higher frequency but lower amplitudes after TCR stimulation that resulted in a reduced TCR-induced rise in $[Ca^{2+}]_i$. Re-expression of Bax in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells restored TCR-dependent Ca^{2+} signaling, indicating that the defect in Ca^{2+} signaling is due to the cell-intrinsic absence of Bax in mature T cells. The decreased Ca^{2+} signaling is sufficient to account for the hypoproliferative phenotype of T cells lacking both Bax and Bak because restoration of Ca^{2+} signals with a Ca^{2+} ionophore restored their ability to proliferate. TCR-dependent $[Ca^{2+}]_i$ signals promoted a rapid increase in mitochondrial NADH production and ROS generation, and the normal coupling of these events is mediated by Bax and Bak. Together, the results establish Bax and Bak as critical regulators of not only T cell apoptosis but also T cell proliferation and demonstrate that Bax and Bak affect T cell activation through control of TCR-dependent Ca^{2+} mobilization and mitochondrial bioenergetics.

The present studies, combined with other lines of evidence, indicate that modulators of T cell apoptosis can also function to limit T cell responsiveness, suggesting that the regulation of proliferation and apoptosis are intrinsically linked in lymphocytes. Mice defective for Fas signaling, which triggers apoptosis of activated T cells, also display defects in T cell proliferation (Scholz et al., 1988; Walsh et al., 1998; Salmena et al., 2003; Chau et al., 2005). Transgenic overexpression of Bcl-2 or Bcl-X_L confers apoptotic resistance to T cells but delays cell-cycle entry after TCR stimulation (Linette et al., 1996; Mazel et al., 1996; Li et al., 2002). The present data demonstrate that the proliferative defect observed in those studies is not simply a consequence of overexpression of Bcl-2 or Bcl-X_L. Loss of Bax and Bak ablates susceptibility to apoptosis and also increases the threshold for T cell activation in vitro and in vivo. As with their effects on T cell viability, Bax and Bak displayed redundant effects on T cell activation, with only their combined loss leading to defects in proliferation. Our data suggest that Bax and Bak function through at least two mechanisms to prevent abnormal lymphocyte expansion: Bax and Bak control the viability of naive and activated T cells through the initiation of mitochondrial-dependent apoptosis, but they also function to prevent unchecked T cell proliferation by modulating ER Ca^{2+} release.

A growing body of evidence suggests that Bcl-2-family proteins, in addition to their role in mitochondrial physiology, can influence cellular Ca^{2+} dynamics through their localization to and action at the ER. Bcl-2 and Bcl-X_L physically associate with the IP_3R and alter its ability to release Ca^{2+} from the ER (Chen et al., 2004; White et al., 2005). Conversely, Bax and Bak can regulate ER Ca^{2+} dynamics (Scorrano et al., 2003; Zong et al., 2003). Here, we demonstrate that defective TCR-dependent Ca^{2+} signaling underlies the T cell proliferation defect of *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells. The integrated TCR- and IP_3-dependent Ca^{2+} signal is reduced in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells because of reduced Ca^{2+} release from the ER, as well as reduced Ca^{2+} influx from outside the cell. However, store-depletion-induced Ca^{2+} influx appears to be normal in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells because the intracellular-dependent $[Ca^{2+}]_i$ signal after thapsigargin treatment was normal. Together, these data suggest that although ER Ca^{2+} content is reduced in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, insufficient depletion of ER Ca^{2+} stores due to low-amplitude $[Ca^{2+}]_i$ spiking in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells may cause reduced influx of Ca^{2+} through the plasma membrane.

The frequency and amplitude of $[Ca^{2+}]_i$ oscillations dictates the signaling properties of Ca^{2+} and promotes different biological outcomes (Berridge et al., 2000). The present results demonstrate that loss of Bax and Bak alters both the magnitude and nature of $[Ca^{2+}]_i$ signals triggered by TCR stimulation. Multiple lines of evidence suggest that proteins of the Bcl-2 family modify Ca^{2+} signaling by altering $[Ca^{2+}]_i$ oscillations. The overexpression of antiapoptotic members of the Bcl-2 family can promote ligand-induced, IP_3R-dependent $[Ca^{2+}]_i$ oscillations (White et al., 2005; Zhong et al., 2006). Mechanistically, the binding of Bcl-X_L to the IP_3R increases its sensitivity to small amounts of IP_3, resulting in greater frequency of ER Ca^{2+} release by receptor stimulation; however, Bcl-X_L also renders the IP_3R more sensitive to inhibition by high Ca^{2+} (White et al., 2005). The consequence is increased frequency of $[Ca^{2+}]_i$ oscillations that have lower amplitudes, resulting in an overall decrease in the integrated $[Ca^{2+}]_i$ response. In *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells, where the activity of Bcl-2 or Bcl-X_L is not opposed by Bax or Bak, we observed a greater frequency of TCR-induced $[Ca^{2+}]_i$ spikes with lower amplitude. The consequence of this change in $[Ca^{2+}]_i$ oscillations is an overall reduction of the

TCR-induced $[Ca^{2+}]_i$ signal, which impacts other Ca^{2+}-dependent events such as NADH production and ROS generation.

TCR ligation activates pathways of cellular metabolism for energy production to meet the increased bioenergetic demands of cell growth and proliferation (Krauss et al., 2001; Frauwirth et al., 2002). How does the shuttling of Ca^{2+} between the ER and mitochondria augment T cell activation and mitochondrial bioenergetics? Ca^{2+} released from the ER is rapidly taken up by mitochondria located in close proximity (Rizzuto et al., 1993). Periodic release of Ca^{2+} from the ER stimulates mitochondrial enzymes of the TCA cycle, leading to the maintenance of efficient oxidative phosphorylation and ATP production (Hajnoczky et al., 1995; Duchen, 2000). However, in response to TCR stimulation, mitochondrial-dependent NADH production doubles. Thus, the Ca^{2+}-induced increase in mitochondrial NADH is greater than that needed to sustain the mitochondrial respiratory chain. The electron transport chain is limited by the electrochemical potential being generated by the increased NADH available as shown by the ability of the electron transport chain to reduce mitochondrial NADH accumulation when an uncoupling agent (such as FCCP) is given. Enhanced NADH production stimulated by TCR-dependent $[Ca^{2+}]_i$ signals results in increased mitochondrial-dependent ROS production, a process that is blunted in *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells because of a reduced overall $[Ca^{2+}]_i$ signal.

What is the importance of Ca^{2+}-dependent ROS generation for T cell activation? Experiments conducted with ROS scavengers have suggested that ROS may be required for T cell proliferation (Chaudhri et al., 1986). TCR-dependent ROS generation can affect mitogen-activated protein kinase (MAPK) activation and early cytokine production in T cells (Devadas et al., 2002; Jackson et al., 2004). Our data suggest that ROS generation is critical for the initiation of cell division after TCR stimulation and that TCR-stimulated ROS is dependent on Ca^{2+} signals regulated by Bax and Bak. Further experiments must be performed to identify the sources and downstream targets of TCR-dependent ROS generation. It should be noted that although T cells lacking functional NADPH oxidase (*Ncf1*$^{-/-}$ and *Cybb*$^{-/-}$, respectively) display reduced TCR-dependent ROS generation, DPI promotes a further reduction of ROS levels, suggesting that TCR-dependent ROS is generated by additional sources (Jackson et al., 2004). Our results suggest that Ca^{2+}-dependent mitochondrial NADH production may contribute to this process by driving increased rates of mitochondrial respiration. ROS is a natural byproduct of mitochondrial respiration when electrons (via NADH) are transferred to O_2 during aerobic ATP generation. In this fashion, mitochondrial-derived ROS may function as a metabolic signal that signifies sufficient energetic conditions exist to support T cell proliferation.

Together, the present data establish that Bax and Bak function as critical regulators of T cell-mediated immunity by integrating diverse signals that regulate T cell proliferation, function, and survival. Bax and Bak function at the ER to maintain sufficient ER Ca^{2+} stores to support TCR-dependent Ca^{2+} signaling pathways. One way Bax and Bak-dependent Ca^{2+} signaling may support T cell proliferation is through rapid effects on mitochondrial energy (NADH) production and ROS generation. Loss of Bax and Bak effectively renders the T cell population inert—T cells are unable to respond to antigenic stimulation but are also unable to die by apoptosis. Consistent with this dual role, the T cell accumulation observed in *Bax*$^{-/-}$, *Bak*$^{-/-}$ animals is entirely composed of small, inert populations of cells (Lindsten et al., 2000). Thus, through their dual effects on proliferation and apoptosis, Bax and Bak control the threshold required for antigen-specific T cell activation and the homeostatic mechanisms that maintain mature T cell viability in vivo.

EXPERIMENTAL PROCEDURES

Mice

Bax$^{-/-}$, *Bak*$^{-/-}$ animals have been described previously (Lindsten et al., 2000). Wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric animals were generated through adoptive transfer of *Bax*$^{+/+}$, *Bak*$^{+/+}$ or *Bax*$^{-/-}$, *Bak*$^{-/-}$ bone marrow or E14-16 fetal liver into lethally irradiated Rag1-deficient mice. Rag1-deficient and C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, MA). All mice were maintained and handled at the University of Pennsylvania according to institutional guidelines.

T Cell Purification

Total or $CD4^+$ T cells were isolated from the spleen and lymph nodes of wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ chimeric animals 8–12 weeks after reconstitution with positive or negative selection kits (StemCell Technologies, Vancouver, B.C.; Miltenyi Biotec Inc., Auburn, CA) and an AutoMACS cell sorter. T cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal-bovine serum, L-glutamine, β-mercaptoethanol, and antibiotics.

T Cell Stimulation and Apoptosis Assays

Purified T cells (0.5 to 1 × 10^6 cells/ml) were stimulated with plate-bound anti-CD3 alone (0.1–10 μg/ml) or in combination with anti-CD28 (1 μg/ml) or ionomycin (250 ng/ml). Alternatively, total splenocytes were activated with soluble anti-CD3 (0.1–1 μg/ml). T cell proliferation was measured by ^{3}[H]-thymidine incorporation (1 μCi/ml) 48 hr after activation or by CFSE dye dilution. T cell ROS production was blocked with diphenylene iodonium (DPI) at 10–100 nM. DPI, BHA, Ebselen, and NAC were obtained from Sigma (St. Louis, MO). For apoptosis assays, T cells were activated for 2 days with plate-bound anti-CD3 and anti-CD28 (5 μg/ml each), washed, and plated in the presence of various apoptotic stimuli (1 × 10^6 cells/ml). For cytokine-withdrawal experiments, T cells were cultured in the presence or absence of recombinant IL-2 (50 U/ml, Peprotech). Viability was measured by propidium iodide (PI) exclusion by flow cytometry.

For Listeria infections, mice were immunized i.v. with a sublethal dose of 5 × 10^4 CFU of recombinant LM expressing OVA (rLmOVA). The construction of this strain has been described previously (Shen et al., 1995; Foulds et al., 2002). Seven days after infection, splenocytes were harvested and OVA-specific T cells detected with MHC class I tetramers ($K^bOVA_{257-264}$). Alternatively, OVA-specific T cells in the peripheral blood of infected mice were detected over time. For intracellular staining, splenocytes were stimulated for 5 hr in IMDM supplemented with 50 U/ml recombinant human IL-2, 1.0 μl/ml GolgiStop (BD PharMingen, San Diego, CA), and the presence or absence of $OVA_{257-264}$ or listeriolysin $O_{190-201}$ ($LLO_{190-201}$) peptide. Intracellular staining was performed with an intracellular staining kit and conjugated antibodies against IFN-γ (clone XMG1.2) (BD PharMingen).

Each experiment was conducted with a minimum of three mice per group.

Retroviral Transduction

Retrovirus for *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cell transduction was produced with established methods (Izon et al., 2001). In brief, 293T cells were cotransfected with pCL-Eco retrovirus packaging vector and either empty hCD8-Mig vector or hCD8-Mig encoding Bax cDNA, and culture supernatant containing high-titer retrovirus was collected 48 hr after transfection. CD4^{+} *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells were purified from spleen and lymph nodes and activated for 24 hr with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (2.5 μg/ml). Activated T cells were cultured in 1 ml retroviral supernatant containing 8 μg/ml polybrene and centrifuged at 2500 rpm for 90 min. Infected T cells were expanded for 3 days in the presence of recombinant IL2 (50–200 U/ml). Transduced T cells were identified with PE- or APC-conjugated antibodies against human CD8α (clone RPA-T8, eBioscience). For some experiments, transduced cells were purified by positive selection with anti-hCD8 microbeads and magnetic sorting (Miltenyi Biotec).

Biochemistry

Purified T cells (2 × 10^6 cells/ml) were stimulated with plate-bound anti-CD3 alone (1 μg/ml) or in combination with anti-CD28 (5 μg/ml) or ionomycin (250 ng/ml). Alternatively, T cells were preincubated with anti-CD3 (10 μg/ml) at 4°C for 30 min and washed twice, and T cell activation was induced at 37°C by addition of anti-hamster-IgG (5 μg/ml, Pearce) to crosslink cell-bound anti-CD3. T cells were lysed with RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing PMSF (1 mM), benzamidine (1 mM), Complete protease inhibitors (Roche), and phosphatase inhibitor cocktail (Sigma). Protein lysates were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with antibodies against ERK (total and pT202/Y204), PLCγ1 (total and pY783) (Cell Signaling Technologies), and Bax (N-20, Santa Cruz Biotechnology). Western blots were resolved with enhanced chemiluminescence (Amersham).

Calcium Measurements

For measurements of $[Ca^{2+}]_i$ dynamics by flow cytometry, T cells were incubated with anti-CD3 (10 μg/ml), probenicid (4 mM), and indo-1 (2 μg/ml) in RPMI containing 1% FCS for 30 min at 30°C, washed, and resuspended in serum-free RPMI. T cell $[Ca^{2+}]_i$ signals were induced by addition of anti-hamster-IgG or addition of IP_3 ester (10 μM) and measured with an LSR flow cytometer (Becton Dickinson). Thapsigargin (Sigma) was added to cells at 1 μM in the presence or absence of 0.5 mM $LaCl_3$ to block Ca^{2+} extrusion across the plasma membrane. For $[Ca^{2+}]_i$ imaging by fluorescence or confocal microscopy, T cells were preincubated with anti-CD3 and loaded with fluo-4, and then they were seeded on coverslips coated with CellTac adhesive (Becton Dickinson). $[Ca^{2+}]_i$ signals were induced by addition of crosslinker. For the study of single-cell $[Ca^{2+}]_i$ dynamics, T cells were loaded with fura-2 and applied to coverslips coated with anti-CD3. Fura-2 was alternately excited at 340 and 380 nm, and the emitted fluorescence filtered at 510 nm was collected and recorded with a CCD-based imaging system running Ultraview software (Perkin Elmer).

NAD(P)H and ROS Measurements

Wild-type or *Bax*$^{-/-}$, *Bak*$^{-/-}$ T cells were preincubated with anti-CD3 (10 μg/ml) for 30 min, washed, and resuspended in Hanks' balanced salt solution (4 × 10^6 cells/ml). T cell activation was induced by addition of anti-hamster-IgG as described above. Autofluorescence of NAD(P)H was monitored at 350/460 nm (excitation/emission) with a multiwavelength excitation, dual-wavelength emission fluorimeter (Delta RAM, PTI, Birmingham, NJ). ROS measurements were performed as described previously (Madesh et al., 2005). In brief, 2′,7′-dichlorofluorescein diacetate (DCF-DA) was added during the last 15 min of preincubation; this was followed by crosslinking with anti-IgG. ROS production was determined by DCF dye oxidation as measured by fluorescence microscopy.

Supplemental Data

Five figures are available at http://www.immunity.com/cgi/content/full/27/2/268/DC1/.

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REFERENCES

Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. *1*, 11–21.

Bidere, N., Su, H.C., and Lenardo, M.J. (2006). Genetic disorders of programmed cell death in the immune system. Annu. Rev. Immunol. *24*, 321–352.

Chao, D.T., Linette, G.P., Boise, L.H., White, L.S., Thompson, C.B., and Korsmeyer, S.J. (1995). Bcl-XL and Bcl-2 repress a common pathway of cell death. J. Exp. Med. *182*, 821–828.

Chau, H., Wong, V., Chen, N.J., Huang, H.L., Lin, W.J., Mirtsos, C., Elford, A.R., Bonnard, M., Wakeham, A., You-Ten, A.I., et al. (2005). Cellular FLICE-inhibitory protein is required for T cell survival and cycling. J. Exp. Med. *202*, 405–413.

Chaudhri, G., Clark, I.A., Hunt, N.H., Cowden, W.B., and Ceredig, R. (1986). Effect of antioxidants on primary alloantigen-induced T cell activation and proliferation. J. Immunol. *137*, 2646–2652.

Chen, R., Valencia, I., Zhong, F., McColl, K.S., Roderick, H.L., Bootman, M.D., Berridge, M.J., Conway, S.J., Holmes, A.B., Mignery, G.A., et al. (2004). Bcl-2 functionally interacts with inositol 1,4,5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5-trisphosphate. J. Cell Biol. *166*, 193–203.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell *8*, 705–711.

Devadas, S., Zaritskaya, L., Rhee, S.G., Oberley, L., and Williams, M.S. (2002). Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: Selective regulation of mitogen-activated protein kinase activation and fas ligand expression. J. Exp. Med. *195*, 59–70.

Donnadieu, E., Bismuth, G., and Trautmann, A. (1992a). Calcium fluxes in T lymphocytes. J. Biol. Chem. *267*, 25864–25872.

Donnadieu, E., Cefai, D., Tan, Y.P., Paresys, G., Bismuth, G., and Trautmann, A. (1992b). Imaging early steps of human T cell activation by antigen-presenting cells. J. Immunol. *148*, 2643–2653.

Duchen, M.R. (2000). Mitochondria and calcium: From cell signalling to cell death. J. Physiol. *529*, 57–68.

Foulds, K.E., Zenewicz, L.A., Shedlock, D.J., Jiang, J., Troy, A.E., and Shen, H. (2002). Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. J. Immunol. *168*, 1528–1532.

Frauwirth, K.A., Riley, J.L., Harris, M.H., Parry, R.V., Rathmell, J.C., Plas, D.R., Elstrom, R.L., June, C.H., and Thompson, C.B. (2002). The CD28 signaling pathway regulates glucose metabolism. Immunity *16*, 769–777.

Hajnoczky, G., Robb-Gaspers, L.D., Seitz, M.B., and Thomas, A.P. (1995). Decoding of cytosolic calcium oscillations in the mitochondria. Cell *82*, 415–424.

Izon, D.J., Punt, J.A., Xu, L., Karnell, F.G., Allman, D., Myung, P.S., Boerth, N.J., Pui, J.C., Koretzky, G.A., and Pear, W.S. (2001). Notch1 regulates maturation of CD4+ and CD8+ thymocytes by modulating TCR signal strength. Immunity *14*, 253–264.

Jackson, S.H., Devadas, S., Kwon, J., Pinto, L.A., and Williams, M.S. (2004). T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. Nat. Immunol. *5*, 818–827.

Krauss, S., Brand, M.D., and Buttgereit, F. (2001). Signaling takes a breath–new quantitative perspectives on bioenergetics and signal transduction. Immunity *15*, 497–502.

Lewis, R.S. (2001). Calcium signaling mechanisms in T lymphocytes. Annu. Rev. Immunol. *19*, 497–521.

Li, C., Fox, C.J., Master, S.R., Bindokas, V.P., Chodosh, L.A., and Thompson, C.B. (2002). Bcl-X(L) affects Ca(2+) homeostasis by altering expression of inositol 1,4,5-trisphosphate receptors. Proc. Natl. Acad. Sci. USA *99*, 9830–9835.

Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., et al. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol. Cell *6*, 1389–1399.

Linette, G.P., Li, Y., Roth, K., and Korsmeyer, S.J. (1996). Cross talk between cell death and cell cycle progression: BCL-2 regulates NFAT-mediated activation. Proc. Natl. Acad. Sci. USA *93*, 9545–9552.

Madesh, M., Hawkins, B.J., Milovanova, T., Bhanumathy, C.D., Joseph, S.K., Ramachandrarao, S.P., Sharma, K., Kurosaki, T., and Fisher, A.B. (2005). Selective role for superoxide in InsP3 receptor-mediated mitochondrial dysfunction and endothelial apoptosis. J. Cell Biol. *170*, 1079–1090.

Marsden, V.S., and Strasser, A. (2003). Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. Annu. Rev. Immunol. *21*, 71–105.

Mazel, S., Burtrum, D., and Petrie, H.T. (1996). Regulation of cell division cycle progression by bcl-2 expression: A potential mechanism for inhibition of programmed cell death. J. Exp. Med. *183*, 2219–2226.

Motoyama, N., Wang, F., Roth, K.A., Sawa, H., Nakayama, K., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., et al. (1995). Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science *267*, 1506–1510.

Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Sawa, H., and Loh, D.Y. (1994). Targeted disruption of Bcl-2 alpha beta in mice: Occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Natl. Acad. Sci. USA *91*, 3700–3704.

Oakes, S.A., Scorrano, L., Opferman, J.T., Bassik, M.C., Nishino, M., Pozzan, T., and Korsmeyer, S.J. (2005). Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA *102*, 105–110.

Ohashi, P.S. (2002). T-cell signalling and autoimmunity: Molecular mechanisms of disease. Nat. Rev. Immunol. *2*, 427–438.

Rathmell, J.C., Lindsten, T., Zong, W.X., Cinalli, R.M., and Thompson, C.B. (2002). Deficiency in Bak and Bax perturbs thymic selection and lymphoid homeostasis. Nat. Immunol. *3*, 932–939.

Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993). Microdomains with high Ca2+ close to IP3-sensitive channels that are sensed by neighboring mitochondria. Science *262*, 744–747.

Salmena, L., Lemmers, B., Hakem, A., Matysiak-Zablocki, E., Murakami, K., Au, P.Y., Berry, D.M., Tamblyn, L., Shehabeldin, A., Migon, E., et al. (2003). Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. Genes Dev. *17*, 883–895.

Scholz, W., Isakov, N., Mally, M.I., Theofilopoulos, A.N., and Altman, A. (1988). Lpr T cell hyporesponsiveness to mitogens linked to deficient receptor-stimulated phosphoinositide hydrolysis. J. Biol. Chem. *263*, 3626–3631.

Scorrano, L., Oakes, S.A., Opferman, J.T., Cheng, E.H., Sorcinelli, M.D., Pozzan, T., and Korsmeyer, S.J. (2003). BAX and BAK regulation of endoplasmic reticulum Ca2+: A control point for apoptosis. Science *300*, 135–139.

Sentman, C.L., Shutter, J.R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S.J. (1991). bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell *67*, 879–888.

Shen, H., Slifka, M.K., Matloubian, M., Jensen, E.R., Ahmed, R., and Miller, J.F. (1995). Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity. Proc. Natl. Acad. Sci. USA *92*, 3987–3991.

Strasser, A., Harris, A.W., and Cory, S. (1991). bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell *67*, 889–899.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., and Dawson, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2(+)-ATPase. Proc. Natl. Acad. Sci. USA *87*, 2466–2470.

Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229–240.

Venkatachalam, K., van Rossum, D.B., Patterson, R.L., Ma, H.T., and Gill, D.L. (2002). The cellular and molecular basis of store-operated calcium entry. Nat. Cell Biol. *4*, E263–E272.

Walsh, C.M., Wen, B.G., Chinnaiyan, A.M., O'Rourke, K., Dixit, V.M., and Hedrick, S.M. (1998). A role for FADD in T cell activation and development. Immunity *8*, 439–449.

Wei, M.C., Lindsten, T., Mootha, V.K., Weiler, S., Gross, A., Ashiya, M., Thompson, C.B., and Korsmeyer, S.J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev. *14*, 2060–2071.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science *292*, 727–730.

White, C., Li, C., Yang, J., Petrenko, N.B., Madesh, M., Thompson, C.B., and Foskett, J.K. (2005). The endoplasmic reticulum gateway to apoptosis by Bcl-X(L) modulation of the InsP3R. Nat. Cell Biol. *7*, 1021–1028.

Zhong, F., Davis, M.C., McColl, K.S., and Distelhorst, C.W. (2006). Bcl-2 differentially regulates Ca2+ signals according to the strength of T cell receptor activation. J. Cell Biol. *172*, 127–137.

Zong, W.X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q.C., Yuan, J., and Thompson, C.B. (2003). Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J. Cell Biol. *162*, 59–69.